REMARKS

This document is submitted in response to the Final Office Action mailed July 22, 2005 ("Office Action"). Applicants have cancelled claims 36, 37, and 40-46.

Applicants have amended claims 18, 21, 38, and 39. Support for the amendment can be found in original claim 18 and the Specification, e.g., at page 1, lines 21-29; page 2, lines 1-8; page 5, lines 7-24; page 7, lines 7-28; page 8, lines 1-18; and page 9, lines 1-25. No new matter has been introduced.

The amendments to claim 18, as well as claims 21, 38, and 39, have been made solely to more particularly point out and distinctly claim the subject matter. The amendments should be entered as they raise no new issues that will require further consideration or search and also do not touch the merits of the application within the meaning of 37 C.F.R. § 1.116(b).

Upon entry of the proposed amendments, claims 18-27, 38, and 39 will be pending. Reconsideration of these claims is requested in view of the following remarks.

Rejections under 35 U.S.C. § 112, first paragraph (written description)

The Office Action maintains its rejection of claims 18, 21, 23, 24, 27, and 36-46 as allegedly lacking written description. Claims 36, 37, and 40-46 have been cancelled. To the extent that the grounds for the rejection may be applied to the remaining claims, Applicants traverse in the remarks below.

I

Claim 18, as amended, explicitly covers an immunogenic composition that contains (1) antigen presenting cells; (2) a purified protein that is a fusion protein consisting essentially of a fragment of human Hsp70 that contains amino acids 481-641 fused to a human antigen unrelated to a heat shock protein ("Hsp 70 fragment fusion protein"); and (3) a pharmaceutically acceptable carrier.

One basis for the rejection appears to be an implicit assumption that for the composition of claim 18 to be immunogenic, the human antigen of the Hsp 70 fragment fusion protein must itself be immunogenic. See, e.g., page 3, lines 19-21; and page 4, line 1. Based on this assumption, the Office Action points out that not all human antigens are immunogenic (i.e., immunogens). It refers to the definitions of "antigen" and "immunogen" provided by Janeway et

al. (page 3, lines 15-20), and opines that the "logic that any antigen can be used in the instant claimed immunogenic composition is flawed" (page 3, lines 20-21; and page 4 line 1). It concludes that as the Specification discloses a single example of a Hsp 70 fragment fusion protein containing an <u>immunogenic human antigen</u> (i.e., PSA), it does not provide a representative number of species to support the immunogenic human antigen genus. See page 4, lines 1-2. Applicants respectfully disagree.

First, the Specification recites a long list of highly diverse <u>immunogenic human antigens</u>, including, e.g., MAGE 1, HER-2/Neu, p53, K-Ras, and EBNA1 (page 2, lines 5-7). <u>See Jäger</u> and Knuth (2001) "Immune responses to tumour antigens: implications for antigen specific immunotherapy of cancer," *J. Clin. Pathol*, 54:669-674, attached hereto as "Exhibit A." Thus, Applicants submit that the Specification did in fact describe a representative number of species of the immunogenic human antigen genus.[1] Further, the nucleotide and amino acid sequences of human Hsp 70 were known in the art at the time of filing. See, e.g., Hunt and Morimoto (1985). It follows that the Specification, taken with the above-described knowledge in the art, would certainly allow a skilled artisan to envision the composition of claim 18 at the time of filing. Accordingly, a skilled artisan would recognize Applicants' possession of the claimed subject matter at the time of filing.

Second, in addition to the Hsp 70 fragment fusion protein, <u>antigen presenting cells</u> are also included in the <u>composition</u> of claim 18. It is submitted that pre-mixing the Hsp 70 fragment fusion protein and antigen presenting cells prior to injection into an animal (e.g., into a tumor area) provides a much greater opportunity for the protein to be displayed by antigen presenting cells thereby triggering an immunogenic response than injecting the Hsp 70 fragment fusion protein <u>by itself</u>. See the Specification, e.g., the paragraph bridging pages 11-12. See also page 1291, entire left column of Armstrong *et al.* (2001) "Cellular immunotherapy for cancer," *BMJ* 323:1289-1293, attached hereto as "Exhibit B." Applicants therefore submit that as the claim 18 composition includes antigen presenting cells, it will be <u>immunogenic</u> even if the Particular human antigen fused to the human Hsp 70 fragment is not immunogenic <u>on its own</u>.

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[1] Applicants further submit that one of ordinary skill in the art could immediately envision an "immunogenic human antigen" even without a single example of an immunogenic human antigen in the Specification, as myriad immunogenic human antigens have already been identified. Thus, a person of ordinary skill would recognize Applicants' intellectual possession of the claimed subject matter at the time of filing.

Based on the remarks presented above, Applicants submit that that one of skill in the art would recognize the composition of claim 18 as immunogenic whether or not the human antigen in the composition is itself immunogenic, and that Applicants had possession of the claimed composition at the time of filing.

II

The Office Action also points out that the meaning of the term "Hsp-70 C-terminal fragment," as previously recited in claim 18, is unclear, and that in any event the Specification lacks written description of this fragment. See page 4, lines 3-7.

Currently amended claim 18 covers an immunogenic composition containing a fusion protein the amino acid sequence of which consists essentially of a fragment of human Hsp 70 containing amino acids 481-641 fused to a human protein antigen unrelated to a heat shock protein.[2] As shown in the Specification, a fragment of human Hsp 70 encoding amino acids 481-641 was amplified by PCR, subcloned, expressed, and purified from *E. coli* as Part of a fusion protein. See, e.g., page 5, lines 16-20, and page 13, lines 15-19. The positions of the primers relative to the encoded amino acid sequence of human Hsp 70 are shown in a figure attached hereto as "Exhibit C." Applicants submit that based on the foregoing remarks, a person of ordinary skill in the art could have readily envisioned the amino acid sequence of amino acids 481-641 encoded by the PCR product disclosed in the Specification. Further, one of ordinary skill in the art could readily envision all possible fragments of human Hsp 70 containing amino acids 481-641. Accordingly, the term "a fragment of human Hsp 70 containing amino acids 481-641" recited in amended claim 18 is clear and meets the written description requirement.

III

The Office Action notes that claim 18 had been amended to include the term "stress protein," as opposed to "heat shock protein." It concludes that this term broadens the claim and constitutes new matter, as it is not defined in the Specification. See page 8, lines 6-18. As currently amended, claim 18 now recites the term "heat shock protein," not "stress protein."

The Office Action also opposes the use of the term "degenerate sequence thereof," previously introduced as an amendment to claim 18, as constituting new matter. See page 8, lines 19-22, and page 9, lines 1-3. Amended claim 18 does not recite this term.

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[2] Examples include a "6X His-tumor antigen-HspC" fusion protein." See, e.g., page 7, lines 7-26.

Applicants submit that as neither of the disputed terms appears in currently amended claim 18, the ground for rejection is mooted.

IV

The Office Action notes that claim 18 had been amended to indicate that the covered composition is "free of any human antigen that is not covalently bound." It concludes that this amendment constitutes new matter, as the Specification does not specifically describe or allude to methods known in the art for purifying Hsp free of non-covalently bound peptides. See page 9, lines 4-18. Applicants respectfully disagree.

Applicants would like to point out that previously amended claim 18, as well as currently amended claim 18, recites an immunogenic composition "free of any human antigen that is not covalently bound." Of note, the Specification discloses that the disclosed Hsp 70 protein or Hsp 70 fragment fusion proteins are purified from bacteria, not human cells. See, e.g., pages 7-10. Applicants therefore submit that based on the teachings of the Specification, the disclosed purified Hsp 70 proteins are, *de facto*, free of any human antigen that is not covalently bound despite Hsp 70's propensity to bind antigens, as noted in the Office Action. Accordingly, the phrase "free of any human antigen that is not covalently bound," recited in claim 18, does not constitute new matter.

V

Based on the foregoing remarks, it is submitted that amended claim 18 meets the written description requirement. For at least the same reasons, claims 21, 23, 24, 27, 38, and 39, dependent from claim 18, also meet the written description requirement.

Rejections under 35 U.S.C. § 112, first paragraph (enablement)

Claims 18-27 and 36-46 were rejected as allegedly lacking enablement on two grounds, which Applicants respectively traverse below.

I

The Office Action notes, with regard to claim 18, that "[t]he specification does not teach a polynucleotide encoding a fragment of HSP-70 flanked by subsequences of both SEQ ID NO:3 and SEQ ID NO:4. While it would be possible using standard molecular biology techniques to generate such a construct, the specification does not have an example of a polynucleotide encoding a fragment of HSP-70 flanked by subsequences of both SEQ ID NO:3 and

SEQ ID NO:4, nor does it provide guidance as to how this would be done or a teaching of why a skilled artisan would wish to generate such a construct." See page 10, lines 18-22; and page 11, lines 1-2.

As currently amended, claim 18 does not recite "a fragment of HSP-70 flanked by subsequences of both SEQ ID NO:3 and SEQ ID NO:4," but instead recites "a purified protein the amino acid sequence of which consists essentially of a fragment of human Hsp 70 containing amino acids 481-641." Further, the Specification teaches primers for amplifying a nucleic acid encoding the just mentioned amino acid range from human Hsp 70, as well as methods for expressing and purifying proteins containing it. See page 5, lines 16-18; and pages 7-10. Applicants therefore submit that the ground for this rejection of claim 18 is mooted.

II

The Office Action takes issue with the fact that claim 18 covered a composition "free of any human antigen that is not covalently bound." See page 11, lines 5-6. It points out that that the art teaches that heat shock proteins inherently bind peptides absent the use of specialized purification techniques. See page 11, lines 6-7. It further notes that the specialized purification techniques are not explicitly taught in the Specification, and therefore concludes that claim 18 is not enabled. See page 11, lines 7-16.

Referring to the same point noted in Part IV of the above discussion of written description, Applicants reiterate that the Specification teaches purification of human Hsp 70 proteins from bacteria. See, e.g., pages 7-10. It is submitted that based on the teachings of the Specification, one of ordinary skill in the art would indeed be able to make the immunogenic composition of claim 18, which is required to be free of any human antigen not covalently bound.

III

Based on the foregoing remarks, Applicants submit that claim 18 is enabled. For at least the same reasons, it is submitted that claims 19-27, 38, and 39, dependent from claim 18, are also enabled.

Rejections under 35 U.S.C. § 112, second paragraph

Claims 18-27 and 36-46 were rejected as allegedly indefinite.

The Office Action again notes that claim 18 covers a composition that includes a Hsp 70 C-terminal fragment encoded by a polynucleotide flanked by two primer sequences, where one of the primer sequences is actually a non-coding (i.e., anti-sense) sequence. See page 12, lines 9-17. Applicants would like to point out that amended claim 18 defines the Hsp 70 C-terminal fragment by amino acid positions rather than by flanking nucleic acid primer sequences, as elaborated above in Part II of the discussion of claim 18 vis-à-vis the written description requirement.

Accordingly, Applicants submit that claim 18, as well as claims 19-27, 38, and 39 dependent from it, is definite.

Rejections under 35 U.S.C. § 103(a)

The Office Action maintains the rejection of claims 18-23, and 36-46 as allegedly obvious over Srivastava ("Srivastava") in view of Suzue et al. ("Suzue").

As amended, claim 18 is drawn to an immunogenic composition containing a purified protein, the amino acid sequence of which consists essentially of the amino acid sequence of a fragment of human Hsp 70, containing amino acids 481-641 of human Hsp 70, fused to a human antigen unrelated to a heat shock protein.

The Office Action of November 3, 2004 points out that Srivastava teaches an immunogenic composition containing: (1) antigen-presenting cells, (2) purified <u>full length</u> human Hsp70, (3) the human antigen PSA, and (4) a pharmaceutically acceptable carrier. It indicates that, the only difference between the composition of Srivastava and the composition of claim 18 is that in the latter a human Hsp 70 is fused, rather than mixed, with a human antigen. See page 5, lines 13-19. It asserts that fusion of a full length Hsp is suggested by Suzue, which discloses an immunogenic full length mycobacterium Hsp 70-antigen fusion protein. On this basis, it alleges that one of ordinary skill in the art would have recognized the inherent advantages of fusing an antigen to a Hsp70, as opposed to mixing it with a Hsp 70, in view of Suzue. It then concludes that claim 18 is rendered obvious by Srivastava and Suzue See page 6, lines 18-22. The current Office Action reiterates this conclusion, and further posits that a person

of ordinary skill in the art would recognize that the advantages of a <u>full length</u> Hsp 70-antigen fusion protein are not specific to the mycobacterial Hsp 70 disclosed by Suzue, and thus would also pertain to full length human Hsp 70. See page 7, lines 3-6. Applicants respectfully disagree with the above conclusion.

Neither Srivastava nor Suzue discloses any <u>fragment</u> of Hsp 70, let alone the specific human Hsp 70 fragment in the fusion protein contained in the claim 18 composition. Importantly, no domain of Hsp 70 (human or mycobacterial) critical to its function in the compositions of Srivastava or Suzue is disclosed in either reference. Applicants therefore submit that one of ordinary skill in the art, without knowing which domains, <u>if any,</u> of a full length Hsp70 were <u>dispensible</u>, would certainly <u>not</u> have been motivated to make a human Hsp 70 fragment fusion protein as contained in the immunogenic composition of claim 18. In this connection, Applicants would like to point out that they developed the composition of claim 18 based on the unexpected finding that purified human Hsp 70 is itself immunogenic. See the Specification, page 16, Table 1. This finding is diametrically opposite of a teaching from Srivastava: "heat shock proteins are not immunogenic per se" (column 2, lines 27-28).

Accordingly, Applicants conclude that claim 18 is not rendered obvious by Srivastava and Suzue. For at least the same reasons, claims 19-23, 38, and 39, dependent from claim 18, are also not rendered obvious by Srivastava and Suzue.

II

The current Office Action also maintains the rejection of claims 18-23 as allegedly obvious over Srivastava in view of Suzue, and further in view of Tong et al. ("Tong").

Applicants submit that as Tong teaches no Hsp 70 fragment whatsoever, it fails to cure the above-stated deficiency of Srivastava and Suzue. In other words, amended claim 18 is not rendered obvious by Srivastava, Suzue, and Tong. For at least the same reasons, neither are claims 19-23, all of which depend from amended claim 18.

III

Based on the foregoing remarks Applicants submit that claim 18, as well as claims 19-23, 38 and 39 dependent from it, is nonobvious over Srivastava and Suzue. Further, claim 18, as well as claims 19-23, is unobvious over Srivastava, Suzue, and Tong. Applicants therefore respectfully request that the rejection be withdrawn.

Claim Objections

The Office Action objects to claims 18-27 and 36-46 on the ground that in these claims the recited sequences do not have corresponding SEQ ID Nos in the Specification. See page 13, lines 2-3. Neither amended claim 18 nor any of the other objected-to claims now recites nucleotide sequences.

The Office Action also objects to claim 18 for a lack of clarity of the recited nucleotide sequence limitations. Amended claim 18 does not recite polynucleotide sequences. Rather, it recites amino acid sequence limitations that are clear, and supported by the Specification.

Accordingly, Applicants submit that the grounds for the objections to claims 18-27 and 36-46 are moot.

CONCLUSION

Based on the remarks set forth above, Applicants submit that all of the pending claims cover allowable subject matter. Early allowance by the Examiner is respectfully solicited.

Enclosed is a $60 check for a petition of a one month extension of time . Please apply any other charges to deposit account 06-1050, referencing attorney docket No. 13886-002001.

Respectfully submitted,

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Immune responses to tumour antigens: implications for antigen specific immunotherapy of cancer

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Abstract

Tumour associated antigens recognised by cellular or humoral effectors of the immune system are potential targets for antigen specific cancer immunotherapy. Different categories of cancer antigens have been identified that induce cytotoxic T lymphocyte (CTL) responses in vitro and in vivo, namely: (1) "cancer testis" (CT) antigens, expressed in different tumours and normal testis, (2) melanocyte differentiation antigens, (3) point mutations of normal genes, (4) self antigens that are overexpressed in malignant tissues, and (5) viral antigens. Clinical studies with peptides and proteins derived from these antigens have been initiated to study the efficacy of inducing specific CTL responses in vivo. Immunological and clinical parameters for the assessment of antigen specific immune responses have been defined—delayed type hypersensitivity (DTH), CTL, autoimmmune, and tumour regression responses. Specific DTH and CTL responses and tumour regression have been observed after the intradermal administration of tumour associated peptides alone. Peptide specific immune reactions were enhanced after using granulocyte macrophage stimulating factor (GM-CSF) as a systemic adjuvant by increasing the frequency of dermal antigen presenting Langerhans cells. Complete tumour regression has been observed in the context of measurable peptide specific CTL. However, in single cases with disease progression after an initial tumour response, either a loss of single antigens targeted by CTL or of the presenting major histocompatibility complex (MHC) class I allele was detected, pointing towards immunisation induced immune escape. Cytokines to modulate antigen and MHC class I expression in vivo are being evaluated to prevent immunoselection. Recently, a new CT antigen, NY-ESO-1, has been identified on the basis of spontaneous antibody responses to tumour associated antigens. NY-ESO-1 appears to be one of the most immunogenic antigens known to date, with spontaneous immune responses observed in 50% of patients with NY-ESO-1 expressing cancers. Clinical studies have been initiated to evaluate the immunogenicity of different NY-ESO-1 constructs to induce both humoral and cellular immune responses in vivo.
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Keywords: tumour antigens; antigen specific T cell response

Spontaneous immune responses against human tumours have been reported in different types of cancer, especially in melanoma and renal cell carcinoma,[1][2] but also in other types of cancer, such as non-small cell lung cancer, bladder carcinoma, and breast cancer, indicating the specific interaction of the immune system with antigenic determinants presented by the tumour.[3][4] The first evidence for antigen specific immune responses arose from studies with cultured melanoma cells, which were lysed by autologous CD8+ T cells in vitro in a major histocompatibility complex (MHC) class I restricted fashion. As a clinical extension of this observation, single patients with metastatic melanoma were vaccinated with irradiated autologous tumour cells for an extended period of time. Two patients (SK-29/MEL-AV and MZ-2/MEL-GH) with refractory metastatic melanoma have been followed by our group since 1978 and 1982, respectively.[5][6] A complete regression of all tumour manifestations was achieved after prolonged immunisation with autologous tumour cells in both patients, which has been maintained for 20 and 15 years, respectively. In a collaborative effort, a systematic search was initiated to identify and characterise the cancer antigens and immune effector mechanisms mediating tumour regression in vivo.[6-8]

Tumour antigens defined by specific T cell responses

CANCER TESTIS ANTIGENS

The specific lysis of cultured tumour cells by autologous cytotoxic T lymphocytes (CTL) in vitro was first observed in melanoma systems.[6] Antigenic peptides presented by MHC class I and II molecules have been identified as the target structures for CTL recognition. The first antigen defined by CTL in the context of histocompatibility leucocyte antigen A1 (HLA-A1) was isolated from a melanoma cell line derived from patient MZ-2, designated MAGE-1.[9] Later, a group of related genes (BAGE, GAGE) was identified, encoding antigens expressed in melanomas and several other tumours, but not in normal tissues except for the testis.[9][10-12] Therefore, antigens of the MAGE pattern of expression are designated

"cancer testis" (CT) antigens. More recently, a new CT antigen, NY-ESO-1, was cloned from an oesophageal cancer by the serological expression cloning method, an approach based on the screening of recombinant tumour cDNA libraries for specific interactions with autologous serum antibodies.[13][14] HLA-A2 binding peptides derived from NY-ESO-1 were found that induce strong CTL responses in vitro.[15] Because NY-ESO-1 and members of the MAGE gene family are frequently expressed in tumours of different histological type, they are attractive targets for antigen specific immunotherapy of cancer.

MELANOCYTE DIFFERENTIATION ANTIGENS

A second group of antigens, first cloned from the SK-MEL-29 system, expressed during melanocyte differentiation was identified as targets for autologous CTL in melanomas.[16-19] Epitopes derived from self antigens such as Melan A/MART-1, tyrosinase, gp100/Pmel17, and gp75/TRP-1 have been found to be targets for CTL and tumour infiltrating lymphocytes (TIL) in the context of HLA-A2.1 and other MHC class I molecules.[20-22] Phase 1 clinical trials in patients with melanoma using antigenic peptides injected intradermally have shown that specific delayed type hypersensitivity (DTH) reactions can be elicited.[23] Granulocyte–macrophage colony stimulating factor (GM-CSF) used as a systemic adjuvant enhanced peptide related DTH reactions in single patients.[24] In contrast to phase I clinical trials with MAGE derived peptides, where peptide specific CTL were rarely identified, the induction of peptide specific CTL was often observed after immunisation with peptides derived from Melan A/MART-1 and tyrosinase.[23][24] Furthermore, objective tumour regression was observed in single patients under continued immunisation.[25-26]

POINT MUTATIONS

Several cancer antigens are defined by point mutations of constitutive cellular proteins, leading to strong CTL responses against tumour cells in patients with cancer or experimental animals.[27-29] In breast cancer, mutations of the p53 and Ras proteins have been reported. Humoral immune responses to the mutated and the wild-type proteins occurring spontaneously in patients with breast, lung, and gastrointestinal cancer have been detected.[30][31] In women with a family history of breast cancer, antibody responses to p53 occur with a higher incidence than in controls (11% v 1%).[30] Because most anti-p53 antibodies detected are of the IgG type, a CD4+ T cell response to p53 can be predicted. In single patients with breast cancer with an overexpression of p53 in primary tumours, a proliferative CD4+ T cell response to wild-type p53 was demonstrated.[32] These findings suggest that immune responses occur after the mutation of oncoproteins. These may also recognise nonmutated portions of the proteins. It is still unknown whether intracellular p53 is also presented at the cancer cell surface or in the extracellular cancer environment to serve as a target

for humoral and/or cellular effectors to mediate tumour regression.

Mutant p53 has been shown to induce specific CTL responses that mediate lysis of the transformed cells in animal models. In a murine sarcoma model, vaccination with p53 peptides combined with interleukin 12 (IL-12) has led to regression of p53 expressing advanced Meth A sarcomas.[33] In many human cancers, the accumulation of wild-type p53 in the cytosol is seen. It is assumed that p53 can be effectively presented by MHC class I molecules to elicit specific CTL responses. Therefore, immune responses against wild-type p53 may be of benefit in the treatment of cancers with p53 accumulation.

Ras mutations involve single amino acid substitutions, mostly at positions 12 and 61. These are less complex than in p53 and thus easier to evaluate. CD4+ and CD8+ T cell responses mediating tumour cell lysis can be induced by immunisation with mutant Ras peptides in animal models.[34] In humans, it remains to be determined whether wild-type or mutant Ras protein is a useful target for active or passive immunotherapy. In a small number of patients with metastatic pancreatic cancer, Ras specific proliferative T cell responses were documented after immunisation with MHC class I restricted Ras peptides.[35]

Other mutation induced antigens defined primarily through CTL recognition—MUM-1 and mutated cyclin dependent kinase 4 (CDK4)—have been shown to be new peptide epitopes presented by MHC class I molecules. It remains to be determined whether these antigens will be useful targets for CTL based vaccines in a larger patient population.[37][38]

OVEREXPRESSED SELF ANTIGENS

Many tumours abundantly express normal self proteins. The most extensively studied self antigens that are targets for active and passive immunotherapy are Melan A/MART-1, a melanocyte differentiation antigen present in melanoma and normal melanocytes, and HER-2/neu, a growth factor receptor overexpressed in 30% of breast and ovarian cancers and a variety of other adenocarcinomas.[10] Immune reactions directed against these antigens may result in the damage of normal tissues. However, preliminary experiences with peptide immunisation in patients with Melan A/MART-1 expressing melanomas have not shown adverse reactions directed to normal tissues, except for the development of vitiligo in single patients.[23] Spontaneous humoral and cellular immune responses in patients with HER-2/neu expressing tumours have been described. They may be amplified by appropriate immunisation strategies, possibly leading to tumour regression.[30]

VIRAL ANTIGENS

Viral diseases are associated with different malignancies in humans—for example, Epstein-Barr virus (EBV) with Burkitts lymphoma,[36] hepatitis B and C viruses (HBV, HCV) with hepatocellular carcinoma,[37][38] human papilloma virus (HPV) with cervical

and anal carcinoma,[38] and human T lymphotropic virus (HTLV) with T cell leukaemia. Independent of whether the viral infection is the oncogenic agent, it was shown that viral antigens are expressed in the associated tumours and can be used as targets for preventive or therapeutic vaccination.[40]

Development of immunotherapeutic strategies

PEPTIDES DERIVED FROM CT ANTIGENS

Peptides derived from MAGE-1 and MAGE-3 have been used alone or combined with different adjuvants—GM-CSF and QS21—for immunisation in HLA-A1 positive patients with MAGE expressing tumours. Tumour regression has been observed in more than 30% of patients with melanoma after immunisation with the MAGE-3 derived, HLA-A1 restricted peptide.[41] However, MAGE-3 specific CTL were not detected in response to the vaccine in these patients.[42] In a subsequent study with systemic GM-CSF to improve antigen presentation by enhancement of CD1a+ dermal Langerhans cells, followed by intradermal administration of MAGE-1 and MAGE-3 peptides, a partial regression of liver and lung metastases was achieved in a patient with melanoma within three months of immunisation (E Jäger *et al*, unpublished data). Correlating with this remarkable clinical development, MAGE-1- and MAGE-3 specific CTL were detected in this patient, and these cells showed an increased frequency after immunisation. Currently, a subsequent phase I study is being initiated to evaluate immune reactions to peptide vaccination in patients with other MAGE expressing carcinomas, such as breast, bladder, non-small cell lung, and head and neck cancer.

MAGE-1 and MAGE-3 specific CTL were repeatedly detected in the peripheral blood of patient MZ2, whose melanoma cell line gave rise to the discovery of the MAGE gene family.[9 10] This suggests that antigen specific CTL may be effective mediators of tumour regression because this patient experienced a complete regression of metastatic, MAGE-1, and MAGE-3 positive melanoma after repeated immunisation with irradiated autologous, MAGE-1/MAGE-3 expressing tumour cells. During the course of continued tumour cell vaccination, increased frequencies of CTL against autologous tumour cells were detected in the peripheral blood of this patient.[43] However, the specificity of CTL responses could not be determined at that time because the structure of the antigenic determinant(s) was unknown. The infrequent detection of CTL against MAGE genes in patients with MAGE expressing melanoma may be a consequence of either a low immunogenicity of MAGE genes, or a frequency of CTL precursors below the level of detection. Different methods for the assessment of MAGE specific CTL responses are being evaluated. A sensitive approach appears to be the ELISPOT assay, an enzyme linked immunosorbent assay that visualises direct antigen–T cell receptor interaction by staining of the spot-like release of interferon γ (IFN-γ) or other cytokines by the T cell, interacting specifically with its target antigen.

TARGETING MELANOCYTE DIFFERENTIATION ANTIGENS

Tumour regression in single patients with melanoma has been achieved after adoptive transfer of TIL lines with specificity for gp100/Pmel17, tyrosinase, and gp75 derived epitopes, suggesting that melanocyte differentiation antigens are tumour rejection antigens.[18 44 45] To study the effects of T cell interactions with melanocyte differentiation antigens in vitro and in vivo we undertook the following investigations. We compared the baseline CTL reactivity against HLA-A2 restricted peptides derived from melan A/MART-1, tyrosinase, and gp100/Pmel17 in HLA-A2 positive patients with melanoma and healthy individuals,[46] and determined CTL responses to melanoma associated peptides injected intradermally as a vaccine in HLA-A2 positive patients with melanoma.[23 34] In addition, we compared changes of expression of melanoma associated antigens and peptide presenting MHC class I molecules in melanoma tissues showing regression or progression in the presence or absence of detectable antigen specific CTL responses in vivo.[47]

First, the spontaneous CTL reactivity against melanoma associated peptides was determined in patients with melanoma and in healthy individuals. Baseline CTL reactivity against the differentiation antigens Melan A/MART-1, tyrosinase, and gp100/Pmel17 is frequently detected in patients with melanoma and in healthy individuals, without significant differences in intensity and frequency of CTL responses.[46 48 49] In healthy individuals, Melan A/MART-1 specific CTL, which lysed Melan A/MART-1 positive melanoma cells were isolated from depigmented skin (vitiligo areas).[50] These findings indicate that CTL responses against self antigens may occur spontaneously, and might be amplified by appropriate vaccination.

Peptides derived from Melan A/MART-1, tyrosinase, or gp100/Pmel17 can induce DTH reactions and specific CD8+ CTL responses after intradermal immunisation. Objective clinical responses were found to be associated with measurable CTL responses to the vaccine. Major toxicity of the vaccine was not observed. However, some patients with favourable clinical picture developed vitiligo.[23 34] In a single patient, a clonal expansion of a Melan A/MART-1 specific T cell receptor Vβ16 was identified in T cell cultures stimulated with Melan A/MART-1 peptide, from Melan A/MART-1 specific DTH reactions, and from vitiligo areas after continued immunisation with Melan A/MART-1 peptide for five years.[51]

Dermal CD1a+ antigen presenting cells (APCs), such as Langerhans cells, can be enhanced and activated by GM-CSF in vivo.[52] Combined administration of melanoma associated peptides and GM-CSF resulted in the amplification of DTH reactions and CD8+ CTL responses. Immunohistochemical characterisation of DTH reactions showed infiltrates of CD4+ and CD8+ T cells and a strong

expression of IL-2 and IFN-γ, suggesting the activation of CD4+ T helper type 1 (Th1) cells and CD8+ CTL by the immunisation peptides presented by MHC class I molecules of dermal APCs.[24]

AUTOLOGOUS AND ALLOGENEIC WHOLE TUMOUR CELL VACCINES

Despite the increasing number of tumour antigens defined in different types of tumours, many investigators have returned to the approach of active immunisation using autologous or allogeneic tumour cells to mount immune responses in patients with cancer without knowing the antigenic repertoire of the individual disease. Tumour cell lysates, irradiated whole tumour cells, and fusion products of tumour cells and autologous or allogeneic dendritic cells have been used for the immunisation of patients with melanoma, breast, and renal cell cancer. Clinical responses of metastatic disease were reported in single cases, but detectable immune responses against the vaccines were difficult to document.[53][54]

Immunoselection of antigen and MHC class I loss variants

Monoclonal antibodies used for immunohistochemical staining of melanocyte differentiation antigens expressed in melanoma tissues are an important prerequisite for studying the microheterogeneity of defined antigens in tumour lesions.[55][56] In HLA-A2 positive patients with melanoma immunised with Melan A/MART-1, tyrosinase, and gp100 derived peptides combined with GM-CSF, we observed after an initial phase of tumour regression in some patients, progressive disease in the presence of detectable peptide specific CTL.[47] When compared with the initially described homogeneous antigen expression, biopsies taken from lesions in the phase of progressive tumour growth showed a highly heterogeneous distribution of antigens in response to increased peptide specific CTL reactivity. Furthermore, a loss of MHC class I molecules, as detected by immunohistochemistry, was found in single cases, and this is an additional mechanism of immune escape from antigen specific immunosurveillance.

Future clinical studies involving antigen specific T cell reactions in patients with cancer will consider the prognostic implication of the heterogeneity of MHC class I and tumour associated antigen expression in tumours for T cell based immunotherapy. Cytokines, such as IFN-γ or IL-12, will be evaluated in future clinical trials to show whether they can modulate the expression of antigens and antigen presenting molecules in tumour tissues.

Immunotherapy in cancer: perspectives

Different types of cancer expressing defined tumour associated antigens may become targets for immunotherapeutic interventions. The growing number of tumour antigens detected and the experience with peptide vaccination in malignant melanoma have set a solid basis for the development of more effective immunotherapeutic strategies in patients with cancer.

CT antigens are thought to be promising targets for specific CTL induced by peptide or protein vaccines. Spontaneous antibody responses to CT antigens detected in the sera of patients with cancer,[57] and the correlation of antibody titres with the course of the disease,[58] suggest the presence of antigen specific CD4+ T cells against peptides presented by MHC class II molecules on the surface of tumour cells.[59] The characterisation of these antigens as targets for CD4+ T cell responses will allow combined immunisation with MHC class I and II binding epitopes, potentially eliciting more effective immune responses.

Targeting viral antigens expressed by different types of cancer, such as cervical and hepatocellular carcinoma, by active immunisation is a strategy currently being evaluated in clinical trials. Although there is some evidence for specific immune responses to the vaccine, major clinical responses have not been achieved yet.[60] Because viral infection is thought to be a tumorigenic factor, immunisation against viral epitopes may have a preventive benefit in stages of premalignancy or even earlier after infection.[61]

Future perspectives of tumour vaccination are focused on the definition of more potent strategies of immunisation. Whole tumour proteins containing multiple, possibly relevant, antigenic epitopes may increase the chance of polyvalent B and T cell activation. Adjuvants might enhance the immunogenicity of peptides and proteins by activating costimulatory factors and mediating the production of cytokines.[62] Dendritic cells loaded with peptides or proteins in vitro, or transduced with the relevant genes, might effectively stimulate both MHC class I and II restricted T cells in vivo.[63][64] Cytokines have been found to play a key role in T cell activation. GM-CSF has been shown to induce long lasting Th1 and CD8+ T cell responses by the efficient induction of dendritic cells in vivo.[65] IL-12 is a potent activator of Th1 and CD8+ T cells. At low doses, it has been shown to mediate complete tumour regression when used as an adjuvant to immunisation with a mutant peptide of p53 in an animal model.[33] The identification of new tumour antigens will provide a broader basis for polyvalent immunisation to prevent the escape of antigen loss variants.[66] As the clinical effectiveness of cancer vaccination becomes more established, antigen specific immunotherapy might be considered as an alternative modality for adjuvant treatment of patients with cancer at high risk for recurrence.

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Clinical review

Science, medicine, and the future

Cellular immunotherapy for cancer

Anne C Armstrong, David Eaton, Joanne C Ewing

During the past decade, our rapidly escalating understanding of immune surveillance and an appreciation of the mechanisms by which tumours escape its notice have led to promising new strategies against cancer. This paper reviews the concepts behind current research into cellular immunotherapy for cancer, presents data from clinical trials, and discusses the potential of this treatment as an adjunct to conventional modes of cancer treatment.

Methods

All three authors are involved in research into cellular immunotherapy and gene therapy. We searched PubMed and Medline databases using the terms "cancer vaccines," "dendritic cells," and "lymphocyte therapy."

The rationale for cellular immunotherapy of cancer

The importance of the interaction between the immune system and cancer cells was recognised in the 1890s when William Coley used streptococcal cultures to treat patients with advanced sarcoma. These attempts to acti-

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Predicted developments

Clinical trials of tumour cell vaccines in patients with minimal residual disease at high risk of relapse

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Translation of cellular approaches into reproducible clinical benefit

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Ability to assay more precisely the clinical and immunological response to cellular treatment

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Definitions of the most potent combinations of effector cells and cytokine enhancers

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vate general immunity led to clinical responses. More recently, antibodies and T cells that identify tumour antigens have been isolated from patients with cancer. It is clear that the immune system is capable of recognising tumour cells.

Cellular immunotherapy consists of giving the patient cells that stimulate antitumour activity in the patient (tumour and dendritic cell vaccines) or that have intrinsic antitumour activity (autologous and allogeneic lymphocytes). The aim is to harness potent immunological weapons to destroy cancer cells.

The immune response to cancer

Cytotoxic T lymphocytes are one of the critical effector cells that are able to lyse tumour cells. Receptors on the surface of T cells recognise antigens presented as peptide fragments on the surface of the class I major histocompatibility complex. Recognition of an antigen by a naive T cell bearing an appropriate T cell receptor is insufficient in itself to trigger activation of the T cell—the antigen must be encountered in conjunction with a costimulatory signal. In the absence of this, T cells become tolerant to the antigen.

Cellular orchestrators of T cell activation are professional antigen presenting cells (dendritic cells) that possess a remarkable ability to stimulate the immune response. These highly specialised cells capture and process antigens that are released during tumour cell breakdown and present them to antigen specific T cells. Once activated, the T cells, including CD4 T helper cells,
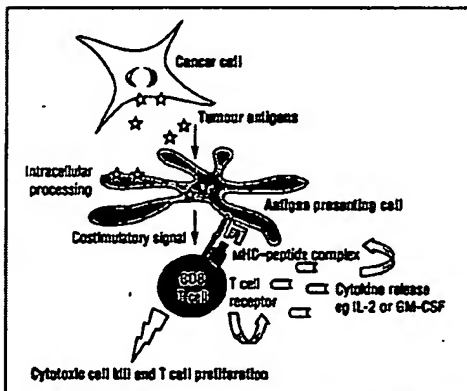

Fig 1 Antitumour immune response. Dendritic cells capture antigens released by cancer cells. After intracellular processing, antigenic peptides are loaded onto major histocompatibility complex (MHC) molecules on the surface of the dendritic cell. Specific T cells encounter these MHC-peptide complexes in conjunction with a costimulatory signal. The activated T cells proliferate and secrete cytokines, resulting in the production of a cascade of immune effector cells (IL-2=interleukin 2; GM-CSF=granulocyte-macrophage colony stimulating factor)

> **Reasons for the failure of immune responses against tumours**
>
> **Impaired tumour recognition by immune cells**
> - Variable expression of tumour antigens
> - Loss of expression of class I major histocompatibility complex, resulting in T cells failing to recognise tumours
>
> **Poor tumour immunogenicity**
> - Many tumour antigens are self antigens and as such are poorly recognised by T cells
> - Lack of costimulatory molecules on tumour cells, which results in failure to stimulate T cells
>
> **Tumour "counterattack"**
> - Tumour cells secrete immunosuppressive cytokines (transforming growth factor β or interleukin 10, for example)
> - Molecules expressed on the surface of tumour cells (for example, Fas ligand) may induce lymphocyte death
> - Evolution of variants of tumour cells that do not express antigens

proliferate and secrete cytokines such as interleukin 2 and granulocyte-macrophage colony stimulating factor. These cytokines are potent stimulators of T cell proliferation and activation and give rise to a cascade of immune effector cells (fig 1).

Despite these highly developed responses, effective immunity against cancer frequently fails to develop—in effect, the immune system becomes blinded to the tumour. The ultimate aim of cellular immunotherapy is to overcome the failed immune response and get the immune system to effectively destroy the tumour cells.

Tumour antigens

As a target for cancer immunotherapy, the ideal tumour antigen is immunogenic and expressed exclusively on tumour cells. Tumour specific antigens include viral antigens and mutated gene products (table). Most known tumour antigens are expressed, to some degree, on normal tissues, and they are therefore "tumour associated" rather than truly tumour specific.

Potential sources of tumour antigens

Category of antigen	Example	Associated tumour
Expression of oncofetal antigens	Carcinoembryonic antigen	Colorectal cancer
	MAGE gene family products*	Melanoma and breast cancer
Tissue specific differentiation antigens	MART-1†	Melanoma
	Glycoprotein gp100	Melanoma
Oncogene/tumour suppressor gene products	p53	Many cancers
	HER-2/neu oncogene	Breast and ovarian cancer
	bcr/abl	Chronic myeloid leukaemia
Viral proteins	Human papilloma virus	Cervical cancer
	Epstein-Barr virus	Burkitt's lymphoma Hodgkin's disease
	Hepatitis B virus	Hepatocellular cancer

*MAGE=melanoma antigen.
†MART-1=melanoma antigen recognised by T cells; also known as Melan A.

Tumour cell vaccines

Whole tumour cell vaccines

Whole tumour cells, rendered safe by irradiation and mixed with an immunological adjuvant, were one of the earliest forms of cellular therapy. This approach avoids the need for tumour antigens to be identified before treatment and allows all of the relevant antigens to be included in the vaccine. Initial clinical studies showed the safety of this approach, with side effects mainly limited to erythematous reactions at the site of the vaccine.

Whole tumour vaccines are now entering phase III trials. One research group vaccinated patients with Dukes's type B and C colorectal cancer with autologous tumour cell vaccines mixed with BCG vaccine.[1] Although there was no difference in overall survival, significant improvements were seen in recurrence free survival in vaccinated patients, with the most benefit seen in patients with the lowest tumour burden. In patients with melanoma and renal cell carcinoma, the results from phase I and II trials have shown possible survival benefits when compared with those from historical controls.[2][3]
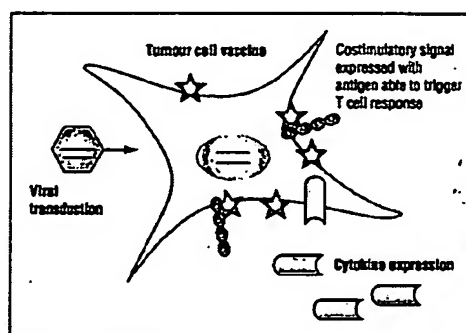

Fig 2 Tumour cell vaccines. Immunogenicity of tumour cell vaccines can be improved by transducing the tumour cell with genes that encode key components of the immune response (cytokines such as granulocyte-macrophage colony stimulating factor (GM-CSF) and costimulatory molecules)

Gene modified vaccines

A more recent approach is the use of vaccines containing genetically modified cells—gene modified vaccines—in which genes encoding key components of the immune response can be introduced into the tumour cells in vitro to increase the immunogenicity of the vaccine (fig 2). The most common gene modified vaccines use cytokines—the cytokine is produced in high concentrations in the vicinity of the tumour cells, where it alters the local immunological environment and enhances the activities of antigen presenting cells and the activation of tumour specific T cells. This approach avoids the side effects associated with systemic treatment with cytokines.

A phase I trial evaluated a tumour vaccine that secretes autologous granulocyte-macrophage colony stimulating factor in patients with metastatic renal cell cancer. This trial provided preliminary evidence of the benefits of this technique, with significant tumour regression in one patient and minimal side effects related to the vaccine.[4]
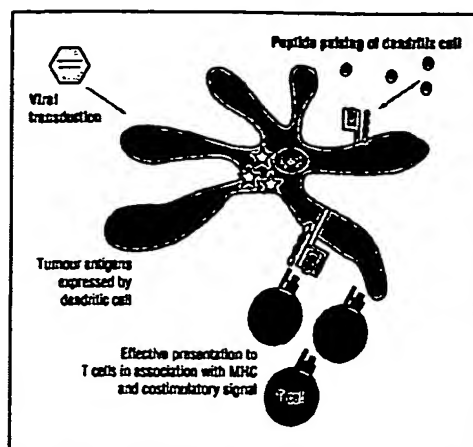
Fig 3 Dendritic cell vaccines. Dendritic cells generated ex vivo from a patient's peripheral blood monocytes or CD34 haemopoietic stem cells can be loaded with tumour antigens and reinfused into the patient with the aim of generating effective antitumour immunity. Loading of antigen can be achieved by a variety of methods, including pulsing cells with antigenic peptides or infecting the cells with recombinant viral vectors (MHC=major histocompatibility complex)

Generation of autologous tumour vaccines is expensive and labour intensive, and not all primary tumours can be expanded to produce enough cells for use in vaccine therapy. An alternative strategy uses gene transduced tumour cell lines as generic vaccines. In these, antigens common to the cell line and the patient's tumour act as targets for an immune response. Tumour antigens are presented to T cells by the host's (the patient's) antigen presenting cells in association with the host's major histocompatibility complex; compatibility of major histocompatibility complex between the patient and the vaccine is therefore unnecessary.[5]

Dendritic cell vaccines

Immunity produced by vaccines depends largely on the efficiency of the antigen presenting cell that initially processes and presents the antigen. Dendritic cells are probably the means by which most vaccines work; they possess an extraordinary capacity to capture and process antigen and contain all that is needed to stimulate T cell immunity, including high levels of major histocompatibility complex, costimulatory molecules, and adhesion molecules. These properties, coupled with the fact that it is now possible to generate, ex vivo, large numbers of functional dendritic cells from a patient's peripheral blood monocytes or CD34 haemopoietic stem cells, have led to considerable interest in the use of dendritic cell vaccines as a means to induce antitumour immunity.

Dendritic cells loaded with tumour antigens in the form of peptide fragments (fig 3), whole antigens, or tumour cell lysates are beginning to enter clinical trials, with some encouraging results. Patients with metastatic melanoma have been vaccinated with dendritic cells loaded with a cocktail of tumour specific peptides or tumour lysates, together with a chemical adjuvant to boost the immune response. In 16 patients, three had

complete responses and two had partial responses.[6] Metastatic renal cell carcinoma has been a target for vaccination with a hybrid cell vaccine consisting of autologous tumour cells fused to dendritic cells. Despite the poor prognosis for such patients, objective clinical responses, including four complete remissions, were seen in 7 of 17 (41%) patients.[7] Ongoing clinical trials are using dendritic cells in renal cell carcinoma, prostate cancer, and melanoma.[8]

Gene therapy techniques can be applied to dendritic cell vaccines; such techniques use recombinant viral vectors that are incapable of replication to provide efficient and reliable means of gene transfer. Genetic material is introduced into dendritic cells to provide them with a renewable source of antigen for presentation; this should lead to more sustained expression of antigen. The expression of viral (and therefore foreign) genes may boost the immune response, but this antiviral immunity primed by dendritic cells may cause the immune system to destroy dendritic cells rapidly in subsequent rounds of immunisation. One solution may be to use viral vectors that do not result in the expression of viral genes, such as retroviruses or "gutless" adenoviral vectors.

Autologous T lymphocyte therapy

The use of interleukin 2 in the treatment of renal cell cancer and melanoma proved that an immunological treatment is capable, in some cases, of inducing long term regression of metastatic tumours. The mechanism by which these remissions occur is believed to be through the stimulatory effects of interleukin 2 on T lymphocytes.[9] Further research showed that tumour infiltrating lymphocytes, isolated from tumour samples
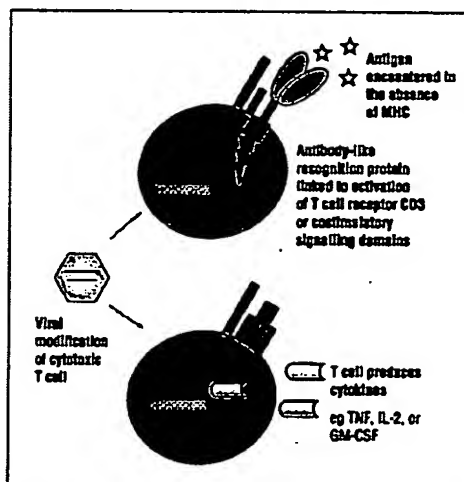
Fig 4 Gene modified T lymphocyte therapy. Lymphocytes can be transduced with genes that encode chimeric receptors consisting of extracellular antitumour antibody fragments linked to intracellular T cell receptors[11] or costimulatory signalling chains.[12] Contact with the tumour leads to proliferation and activation of the antigen specific T cells. Lymphocyte survival and antitumour efficacy may also be improved by the use of genes encoding various cytokines (MHC=major histocompatibility complex; TNF=tumour necrosis factor; IL-2=interleukin 2; GM-CSF=granulocyte-macrophage colony stimulating factor)

Fig 5 Adoptive immunotherapy with cytotoxic T lymphocytes specific for minor histocompatibility antigens restricted to the haemopoietic system. Responder lymphocytes from the allogeneic donor (who may be a sibling or unrelated matched donor) are stimulated by dendritic cells pulsed with antigens expressed specifically by haemopoietic cells from the recipient. This leads to the expansion of a specific T cell clone that is able to lyse recipient haemopoietic cells but is unable to a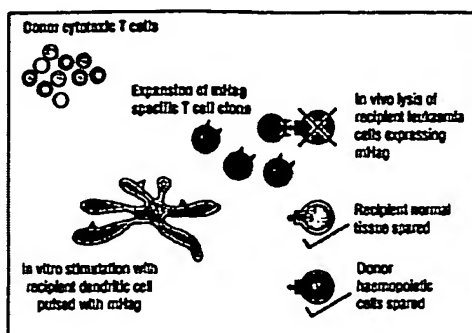ttack the recipient tissues, which are susceptible to graft versus host disease. (mHag=minor histocompatibility antigens)

and grown in interleukin 2, could also induce remissions in these disease groups. Disappointingly, in patients receiving interleukin 2, the infusion of these cells did not improve response or survival rates significantly compared with those receiving interleukin 2 alone.[10]

More recently, advances in the ex vivo use of gene transfer technology to genetically modify lymphocytes have made it possible to increase their effectiveness. One strategy involves fusing the antigen recognition domains of specific antitumour antibodies with intracellular T cell receptor signalling chains to form "chimeric" T cell receptors (fig 4). Cytotoxic T lymphocytes modified to express such receptors are specifically activated on contact with tumour antigen, without the need for tumour expression of major histocompatibility complex. T cells genetically modified in this way have been used successfully to treat human ovarian cancer cells in immunodeficient mice,[11] and clinical trials are ongoing.

Other approaches being studied include increasing antitumour efficacy by modifying lymphocytes to secrete antitumour cytokines, such as tumour necrosis factor, and improving in vivo T cell survival through the autocrine production of growth factors such as interleukins.

Allogeneic lymphocyte therapy

A potent graft versus leukaemia effect may be mediated by donor T cells that recognise disparities between donor's and host's tissue histocompatibility antigens as well as tumour antigens. Infusions of allogeneic donor leucocytes led to clinical responses in 60-80% of patients with chronic myeloid leukaemia who had relapsed after allogeneic transplantation. Recent reports suggest that a graft versus tumour response may be successfully induced against solid tumours such as renal cell carcinoma.[15]

Unfortunately, use of allogeneic lymphocytes is frequently accompanied by graft versus host disease, in which donor T cells recognise the host tissue as "foreign." Novel approaches are being used to separate the graft versus leukaemia effect from the graft versus

host effect, which should make giving donor leucocytes safer. Donor lymphocytes can be genetically modified to express genes that sensitise cells to specific drugs that can be administered to trigger cell death. This may confer the ability to eliminate effector T cells in the instance of toxic graft versus host disease.[14]

Specifically selected allogeneic donor cytotoxic T lymphocytes offer the prospect of an antileukaemia effect in the absence of graft versus host disease. One exciting approach may be the expansion ex vivo of those allogeneic cytotoxic T lymphocytes that are able to selectively recognise those minor histocompatibility antigens whose expression is restricted to recipient haemopoietic (and therefore leukaemic) cells (fig 5).[15] The Wilms's tumour gene WT1 is expressed at increased levels on the blast cells of patients with acute myeloid leukaemia and chronic myelogenous leukaemia. Current approaches are looking at the potential for exploiting WT1 as a target molecule, in order to selectively direct cytotoxic T lymphocytes against leukaemic blast cells.[16]

Limitations of cellular therapy

One concern with cellular immunotherapy is the induction of autoimmunity—vitiligo developed in 20% of melanoma patients who responded to interleukin 2.[17] Other evidence of autoimmune disease has not been seen in any of the cancer vaccine trials to date, but is a possibility. Inducing autoimmunity against organs for which replacement therapy is available, such as the pancreas, may be acceptable to patients who otherwise face the possibility of dying from their disease, but a more widespread autoimmune reaction could limit the use of some cancer vaccines.

We have discussed small pilot studies performed in specialist units, but it is important to prove clinical benefit in large, randomised studies. Cellular therapy is expensive, time consuming, and complex, and adopting this approach on a large scale will be challenging.

The future

Most clinical trials to date have vaccinated patients with advanced disease. These patients will have some degree of immunosuppression, from the cancer itself and as a result of previous treatment. Immunisation strategies are likely to be most beneficial when applied to patients with minimal levels of disease and tumour types known to be particularly immunogenic, such as melanoma and renal cell carcinoma. Safety issues must be evaluated in patients where no conventional treatment is proved to be successful; however, as we move from the realm of pilot studies, it will be critical to design future clinical trials to tackle the subject of residual disease burden, which may occur after surgery. Preliminary research suggests that these therapies will be less toxic than more conventional modes of treatment.

Cellular therapy is a rapidly evolving field, with incremental technological advances in cellular manipulation and genetic modification. As we attain a deeper understanding of the power of the immune response, we may be able to exploit this system and use it as a platform on which to build a successful therapeutic strategy to fight cancer.

Additional educational resources

Websites

CancerNet (www.cancernet.nci.nih.gov)
Information about cancer vaccine trials that are
currently recruiting

Gene therapy advisory committee (www.doh.gov.uk/
genetics/gtac)

UK gene therapy trials approved by the gene therapy
advisory committee

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Miguel de Cervantes, hydropsy, and Thomas Sydenham

One day during the spring of 1616, as three friends quickly rode their horses from Esquivias to Madrid, a student hurried behind on a donkey and begged them to slow down. As they did so one of the gentlemen told the student that "Señor Miguel de Cervantes' fast mount" was the reason for their swift pace. The student, recognising Cervantes' paralysed hand, addressed him with admiration and devotion. The four resumed their journey and Cervantes wrote, "We talked about my disease," and quoted the student saying. "Your malady is hydropsy, something which cannot heal even if you were to drink as much sweet water as there is salt water in the entire ocean ... Señor Cervantes, limit your drinking, without forgetting to eat—this will help you without having to take any remedy." Cervantes confirmed, "Many others have said so, but I can't avoid trying to quench my thirst, as though drinking were the very reason for which I was born," and he added, "My life is coming to an end, to go by my pulses—they'll stop beating on Sunday and I'll die."

Miguel de Cervantes Saavedra died four days after writing the above conversation in the prologue to his posthumous book *Los trabajos de Persiles y Sigismunda* (1617). He died in Madrid at the age of 68 on Saturday 23 April 1616 (on the same day as William Shakespeare). What caused his death? Does his biography give any clue to his last illness?

In 1569, aged 22, Cervantes contracted malaria near Rome and malaria still troubled him at Lepanto on 7 September 1571, when, despite high fever and his fellow sailors' advice to rest, Cervantes took part in the battle and was wounded. Two gunshots hit his chest, one crippled his left hand, hence his nickname "*el manco* (one handed) *sano* (unamputated)." His wounds had not yet healed when he was captured on 26 September 1575 off Aigues Mortes by pirates while sailing from Naples to Spain. He was kept prisoner in Algeria for five years, where, after each attempt at escaping, he was flogged and was once kept chained "from head to feet" for five months.[1]

If his medical history cannot explain Cervantes' hydropsy, can his writings do so?

Cervantes treats medical questions in his *Don Quijote de la Mancha* with such exactness that some have wondered whether he was a physician.[1] In his works he names over 100 medicinal plants and his description of Don Quixote's psychological development earned him the nickname "Raphael of medicine." Cervantes uses the term hydropsical—not hydropsy—once in his *Don Quijote*, meaning ascitic.

But did hydropsy mean anything else in Cervantes' time? The *Tesoro de la Lengua Castellana o Española*, published in Madrid in 1611, defines hydropsy as "a disease due to watery humour that swells the body" and adds that it also means avarice because "patients with hydropsy can drink as much as they like, they'll never quench their thirst, like misers, no matter how much they gain, cannot appease their greed."

Did Cervantes have polydipsia? Did he have diabetes, ascites, uraemia, anasarca, or heart failure? Heart disease seemed "logical" a century ago "since he had such a good [literally large] heart."[1]

The enigma remains. But its solution does not matter. More important is Thomas Sydenham's (the English Hippocrates') answer to his disciple Richard Blackmore's question what to read to become a better doctor: "read *Don Quixote*."

Bruno Simini *staff anaesthetist, Ospedale Generale Provinciale, Lucca, Italy*

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Positions of Forward and Reverse "Hsp C-terminal" Primer Sequences in the Human Hsp 70 Sequence (See nucleotide positions encoding amino acids 481-641) Sequence is from GenBank NM_005345

```
1      atg gcc aaa gcc gcg gcg atc ggc atc gac ctg ggc acc acc tac   45
1       M   A   K   A   A   A   I   G   I   D   L   G   T   T   Y     15

46     tcc tgc gtg ggg gtg ttc caa cac ggc aag gtg gag atc atc gcc   90
16      S   C   V   G   V   F   Q   H   G   K   V   E   I   I   A     30

91     aac gac cag ggc aac cgc acc acc ccc agc tac gtg gcc ttc acg.  135
31      N   D   Q   G   N   R   T   T   P   S   Y   V   A   F   T     45

136    gac acc gag cgg ctc atc ggg gat gcg gcc aag aac cag gtg gcg   180
46      D   T   E   R   L   I   G   D   A   K   N   Q   V   A         60

181    ctg aac ccg cag aac acc gtg ttt gac gcg aag cgg ctg atc ggc   225
61      L   N   P   Q   N   T   V   F   D   A   K   R   L   I   G     75

226    cgc aag ttc ggc gac ccg gtg gtg cag tcg gac atg aag cac tgg   270
76      R   K   F   G   D   P   V   V  ·Q   S   D   M   K   H   W     90

271    cct ttc cag gtg atc aac gac gga gac aag ccc aag gtg cag gtg   315
91      P   F   Q   V   I   N   D   G   D   K   P   K   V   Q   V·    105

316    agc tac aag ggg gac acc aag gca ttc tac ccc gag gag atc tcg   360
106     S   Y   K   G   D   T   K   A   F   Y   P   E   E   I   S     120

361    tcc atg gtg ctg acc aag atg aag gag atc gcc gag gcg tac ctg   405
121     S   M   V   L   T   K   M   K   E   I   A   E   A   Y   L     135

406    ggc tac ccg gtg acc aac gcg gtg atc acc gtg ccg gcc tac ttc   450
136     G   Y   P   V   T   N   A   V   I   T   V   P   A   Y   F     150

451    aac gac tcg cag cgc cag gcc acc aag gat gcg ggt gtg atc gcg   495
151     N   D   S   Q   R   Q   A   T   K   D   A   G   V   I   A     165

496    ggg ctc aac gtg ctg cgg atc atc aac gag ccc acg gcc gcc gcc   540
166     G   L   N   V   L   R   I   I   N   E   P   T   A   A   A     180

541    atc gcc tac ggc ctg gac aga acg ggc aag ggg gag cgc aac gtg   585
181     I   A   Y   G   L   D   R   T   G   K   G   E   R   N   V     195

586    ctc atc ttt gac ctg ggc ggg ggc acc ttc gac gtg tcc atc ctg   630
196     L   I   F   D   L   G   G   G   T   F   D   V   S   I   L     210

631    acg atc gac gac ggc atc ttc gag gtg aag gcc acg gcc ggg gac   675
211     T   I   D   D   G   I   F   E   V   K   A   T   A   G   D     225

676    acc cac ctg ggt ggg gag gac ttt gac aac agg ctg gtg aac cac   720
226     T   H   L   G   G   E   D   F   D   N   R   L   V   N   H     240

721    ttc gtg gag gag ttc aag aga aaa cac aag aag gac atc agc cag   765
241     F   V   E   E   F   K   R   K   H   K   K   D   I   S   Q     255

766    aac aag cga gcc gtg agg cgg ctg cgc acc gcc tgc gag agg gcc   810
256     N   K   R   A   V   R   R   L   R   T   A   C   E   R   A     270

811    aag agg acc ctg tcg tcc agc acc cag gcc agc ctg gag atc gac   855
271     K   R   T   L   S   S   S   T   Q   A   S   L   E   I   D     285

856    tcc ctg ttt gag ggc atc gac ttc tac acg tcc atc acc agg gcg   900
```

```
286    S   L   F   E   G   I   D   F   Y   T   S   I  .T   R   A    300


901  agg ttc gag gag ctg tgc tcc gac ctg ttc cga agc acc ctg gag  945
301    R   F   E   E   L   C   S   D   L   F   R   S   T   L   E    315

946  ccc gtg gag aag gct ctg cgc gac gcc aag ctg gac aag gcc cag  990
316    P   V   E   K   A   L   R   D   A   K   L   D   K   A   Q    330

991  att cac gac ctg gtc ctg gtc ggg ggc tcc acc cgc atc ccc aag 1035
331    I   H   D   L   V   L   V   G   G   S   T   R   I   P   K    345

1036 gtg cag aag ctg ctg cag gac ttc ttc aac ggg cgc gac ctg aac 1080
346    V   Q   K   L   L   Q   D   F   F   N   G   R   D   L   N    360

1081 aag agc atc aac ccc gac gag gct gtg gcc tac ggg gcg gcg gtg 1125
361    K   S   I   N   P   D   E   A   V   A   Y   G   A   A   V    375

1126 cag gcg gcc atc ctg atg ggg gac aag tcc gag aac gtg cag gac 1170
376    Q   A   A   I   L   M   G   D   K   S   E   N   V   Q   D    390

1171 ctg ctg ctg ctg gac gtg gct ccc ctg tcg ctg ggg ctg gag acg 1215
391    L   L   L   L   D   V   A   P   L   S   L   G   L   E   T    405

1216 gcc gga ggc gtg atg act gcc ctg atc aag cgc aac tcc acc atc 1260
406    A   G   G   V   M   T   A   L   I   K   R   N   S   T   I    420

1261 ccc acc aag cag acg cag atc ttc acc acc tac tcc gac aac caa 1305
421    P   T   K   Q   T   Q   I   F   T   T   Y   S   D   N   Q    435

1306 ccc ggg gtg ctg atc cag gtg tac gag ggc gag agg gcc atg acg 1350
436    P   G   V   L   I   Q   V   Y   E   G   E   R   A   M   T    450

1351 aaa gac aac aat ctg ttg ggg cgc ttc gag ctg agc ggc atc cct 1395
451    K   D   N   N   L   L   G   R   F   E   L   S   G   I   P    465

1396 ccg gcc ccc agg ggc gtg ccc cag atc gag gtg acc ttc gac atc 1440
466    P   A   P   R   G   V   P   Q   I   E   V   T   F   D   I    480
```

Sequence from Hsp C'-specific Forward primer matching human Hsp 70 sequence

```
     5--gat gcc aac ggc atc ctg aac-3'--->
1441 gat gcc aac ggc atc ctg aac gtc acg gcc acg gac aag agc acc 1485
481    D   A   N   G   I   L   N   V   T   A   T   D   K   S   T    495

1486 ggc aag gcc aac aag atc acc atc acc aac gac aag ggc cgc ctg 1530
496    G   K   A   N   K.  I   T   I   T   N   D   K   G   R   L    510

1531 agc aag gag gag atc gag cgc atg gtg cag gag gcg gag aag tac 1575
511    S   K   E   E   I   E   R   M   V   Q   E   A   E   K   Y    525

1576 aaa gcg gag gac gag gtg cag cgc gag agg gtg tca gcc aag aac 1620
526    K   A   E   D   E   V   Q   R   E   R   V   S   A   K   N    540

1621 gcc ctg gag tcc tac gcc ttc aac atg aag agc gcc gtg gag gat 1665
541    A   L   E   S   Y   A   F   N   M   K   S   A   V   E   D    555

1666 gag ggg ctc aag ggc aag atc agc gag gcc gac aag aag aag gtg 1710
556    E   G   L   K   G   K   I   S   E   A   D   K   K   K   V    570

1711 ctg gac aag tgt caa gag gtc atc tcg tgg ctg gac gcc aac acc 1755
571    L   D   K   C   Q   E   V   I   S   W   L   D   A   N   T    585
```

```
1756   ttg gcc gag aag gac gag ttt gag cac aag agg aag gag ctg gag   1800
586     L   A   E   K   D   E   F   E   H   K   R   K   E   L   E    600

1801   cag gtg tgt aac ccc atc atc agc gga ctg tac cag ggt gcc ggt   1845
601     Q   V   C   N   P   I   I   S   G   L   Y   Q   G   A   G    615

1846   ggt ccc ggg cct ggg ggc ttc ggg gct cag ggt ccc aag gga ggg   1890
616     G   P   G   P   G   G   F   G   A   Q   G   P   K   G   G    630
```

Sequence from Hsp C'-specific Reverse primer matching Human Hsp 70 sequence

```
                <---3'-g tgg taa ctc ctc cat cta atc---5'
1891   tct ggg tca ggc ccc acc att gag gag gta gat tag   1926
631     S   G   S   G   P   T   I   E   E   V   D   *
```

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